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A MODIFIED METHOD FOR THE DETERMINATION OF THE AZORUBIN BINDING CAPACITY OF ALBUMIN IN SMALL AMOUNTS OF SERUM

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Report No. 63

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ABSTRACT

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OBJECT

Two ml of serum or protein solution are needed for the chromatographic determination of the azorubin-binding capacity (ABC) of serum albumin. In applying this method to studies of the effect of stress on small laboratory animals it became desirable to use smaller amounts of serum. In the course of the necessary modifications in the method, certain features were investigated in order to verify the validity of the procedure.

RESULTS

A modified method has been described for the determination of the ABC, using only 1 ml of serum or protein solution. The experimental conditions for this procedure have been studied. The new method gives results equally as valid as those of the previously published procedure which necessitated larger amounts of serum. (1)

A process for a graded deactivation of anionotropic aluminum oxide has been developed.

Attempts to replace the anionotropic aluminum oxide by synthetic anion exchangers were unsuccessful. This is believed to be due to the slow diffusion of large anions, such as axorubin, into the solid resins. This process influences the degree of retention. Employing anionotropic aluminum oxide, the anions are mainly adsorbed at the active centers of the surface. The retention therefore is more uniform.

In studies on the competitive reaction between anionotropic aluminum oxide and albumin for azorubin, a quantitative relationship has been established between the albumin concentration and the specific ABC values.

The ABC of a serum albumin solution is not influenced by the addition of a non-binding protein (gamma globulin).

The calibration curves for azorubin bound to serum albumin of different species were found to be different. The ABC values of bovine serum differ from those of other species.

CONCLUSIONS

The present chromatographic method for a comparative determination of the ABC of serum albumin can be applied to studies on small laboratory animals. In the interpretation of the results, the influence of low albumin concentrations on the specific ABC must be considered.

RECOMMENDATIONS

The modified method can be used in determining the specific ABC of the serum of animals subjected to various forms of stress as well as experimental liver damage.

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A MODIFIED METHOD FOR THE DETERMINATION OF THE AZORUBIN-BINDING CAPACITY OF ALBUMIN IN SMALL AMOUNTS OF SERUM

I. INTRODUCTION

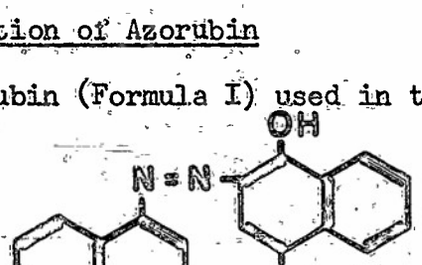
Previous studies have demonstrated that the capacity of human serum albumin to bind the anionic dye azorubin is decreased in certain pathological conditions (1, 2). The "azorubin-binding capacity" (ABC) was determined by a chromatographic method which separates the free dye from the albumin-bound azorubin by adsorption on anionotropic aluminum oxide. The procedure can be used for sera as well as for other protein solutions.

Two ml of serum are needed for the blank and each run, i.e., a total of 8 ml are required if the analysis is made in triplicate. For studies using small laboratory animals it was desirable to reduce the amount of serum required. A modified procedure which needs only half the amount of serum has been worked out and is described in the present report. In order to verify the validity of the modified method, experiments have been carried out (a) on the fractional distribution of azorubin in the chromatographic filtrate and (b) on the competitive reaction between the aluminum oxide and albumin for azorubin.

II. PROCEDURE AND RESULTS

A. Purification of Azorubin

The azorubin (Formula I) used in the chromatographic studies was



I

obtained from the Hartman-Leddon Company, Philadelphia, Pa. (C. I. No. 179). It was recrystallized once from water made acid to congo red by the addition of HCl, and three times from an aqueous solution by the addition of 3 to 4 volumes of acetone. The dye then was adsorbed on aluminum oxide in a chromatographic column, eluted with water and finally crystallized from a water-acetone solution.

The absorption curve of the purified azorubin, dissolved in $\frac{M}{15}$ phosphate at pH 7.7, showed a maximum at 515 mμ. The original commercial azorubin contained a colorless material to the extent of about 20% as shown by a comparison with the extinction coefficients of the purified preparation.

B. Studies on Deactivation of Anionotropic Aluminum Oxide

It was found that an anionotropic aluminum oxide prepared according to the previous procedure (1) from Reagent Aluminum Oxide (Merck and Company, Rahway, N. J., No. 71707, suitable for chromatographic adsorption), showed greater activity than the material used previously (1, 2). Possibilities of

deactivating anionotropic aluminum oxide were therefore investigated. The activity was tested by determining the specific ABC values of human albumin* solutions (See Section D).

The anionotropic aluminum oxide was refluxed with distilled water for various periods of time. In several cases a slight decrease of activity was observed after refluxing for many hours, but this result could not be obtained with regularity. A marked lowering of the anionotropic activity was found after boiling in acetate buffer. The use of buffer solutions of increasing pH resulted in a gradual decrease of the activity (Table 1). The positions of the red adsorption zones of azorubin in the chromatographic columns were practically the same as with the original anionotropic adsorbents.

TABLE 1

DEACTIVATION OF ANIONOTROPIC ALUMINUM OXIDE BY REFLUXING WITH ACETATE BUFFER SOLUTIONS

3 gm of anionotropic Al_2O_3 (Prep. 6-7-51) were refluxed for 1 hour with 50 ml of $\frac{M}{20}$ acetate buffer.

Test mixture: 1.0 ml 3.08% Human Albumin in Krebs Buffer (pH 7.8) + 0.25 ml 0.5% Azorubin in 0.6% NaCl (pH 7.8).

pH of Acetate Buffer		ABC Observed	Specific ABC
Before Experiment	After Experiment	mg %	10^{-5} Mol Azorubin per gm Albumin
(Control)	-	30.1	2.44
4.0	3.6	35.4	2.87
5.0	4.9	39.6	3.21
6.0	5.7	46.9	3.80
7.0	5.8	48.8	3.95

* We are greatly indebted to Dr. J. T. Edsall and Dr. L. D. Wojcik, University Laboratory of Physical Chemistry, Harvard University for kindly supplying the human albumin and gamma globulin preparations used in these studies.

C. Studies on Adsorption by Anion Exchangers

The reproducibility of the method described in Section D is dependent on the activity of the anionotropic aluminum oxide. It was found that slightly varying activities may be encountered, even if the anionotropic adsorbent is prepared from the same starting material. Various resinous anion exchangers were tried in an attempt to find an adsorbent of constant activity. The data in Table 2 show that most of the exchangers used adsorb azorubin under the conditions employed. Resins of comparatively large particle size and high speed of flow (Nos. 5, 10, 12) did not retain all the dye used. This is consistent with the concept that the adsorption of anions by exchangers of this type is governed by the diffusion into the resin particles (3), the diffusion rate of an anion as large as azorubin (M. W. = 502) being very small compared to the anions of ordinary acids.

As may be seen from Table 3 (experiments 1 and 3), some resinous anion exchangers seemingly can be used in the same manner as anionotropic Al_2O_3, to separate free and albumin-bound azorubin. Other resins, however, detach the dye completely from the protein (experiments 2 and 4). Evidence for a true separation of azorubin from albumin is indicated by the finding that the albumin concentrations in the last part of the filtrate (see Section D) were about the same in all experiments shown in Table 3.

Experiments 5 to 7 of Table 3 indicate that the degree of detaching azorubin from albumin depends on the size of the resin particles. The exchanger Ionac A-293M was ground and separated into 3 fractions according to particle size. As may be seen from Table 3, the azorubin concentrations in the last part of the chromatographic filtrate changed with the particle size of the resins.

It appears from these observations that the synthetic anion exchangers are not suitable for a chromatographic determination of ABC. Even if material of uniform size were used, the rate of flow would greatly influence the results because of the comparatively slow diffusion of azorubin into the resin particles.

D. A Modified Method for the Determination of the Azorubin-Binding Capacity in Small Amounts of Serum

In previous studies, 2 ml of serum were needed for one chromatographic run. This amount was changed to 1 ml in the modified procedure. For greater reliability, the chromatographic analysis is generally done in triplicate, or at least in duplicate, and a blank run is made to compensate for any color of the serum (hemolysis). Therefore a total of 3 or 4 ml of serum are required.

The chromatographic tubes employed are about 20 cm long, have an inner diameter of 5 mm and end in a 0.5 mm capillary of 2 to 3 cm length. A funnel is sealed to the upper end. The columns are prepared using 500 mg of anionotropic aluminum oxide suspended in double-distilled water. The length of the column is about 25 mm. For each run a mixture of 1 ml of serum and 0.25 ml of an azorubin solution (mostly 0.5%) in 0.6% sodium chloride (pH 7.8) is prepared at least 30 minutes prior to the analysis. The azorubin

TABLE 2
CHROMATOGRAPHIC ADSORPTION OF
AZORUBIN BY ANION EXCHANGERS

Inner diameter of chromatographic tube used for all experiments: 5 mm.
Azorubin solution: 1.25 ml of a 50 mg % solution of commercial azorubin (Hartman-Ledden Co., Philadelphia, Pa.) in 0.6% NaCl, pH adjusted to 7.8.
The adsorbents were ground for 3-4 minutes in distilled water, rinsed 3 times with water, and washed into the chromatographic tube.

No	Anion Exchanger	Obtained from	Color of Column	Length of Column mm	Time of Drainage for 1.25 ml		Adsorption in mm from top	Filtrate
					Min.	Sec.		
1	Deacidite	1	Dark red-brown	50	4	40	0-5	Colorless
2	Ionac A-293M	1	White	48	10	34	0-2	Colorless
3	Ionac A-300	1	Yellow	54	12	9	0-1	Colorless
4	Permutit 5	1	Yellow	45	1	7	0-16	Colorless
5	Amberlite IR-45 Lot No. 3631	2	Yellow	52	1	15	0-52	Pink
6	Amberlite IRA-410 Lot No. 35204	2	Yellow	50	5	25	0-3	Colorless
7	Amberlite IRA-400 A-624/5	3	Yellow	48	2	44	0-9	Colorless
8	Amberlite IR-4B A-624/1	3	Dark orange speckled with black	43	1	48	0-19	Colorless
9	Duolite A-2	4	Speckled brown-white	54	5	22	0-0.5	Colorless
10	Duolite A-70	4	Dark red	42	-	25	0-42	Red
11	Nalcite SAR	5	Pale yellow	50	1	45	0-25	Colorless
12	Nalcite WBR	5	Yellow	43	-	50	0-43	Red
13	Dowex 1 L2330-15	6	Pale yellow	53	8	36	0-3	Colorless

*1) The Permutit Co., New York 18, N. Y. 4) Chemical Process Co., Redwood City, Cal.
2) Rohm and Haas Co., Philadelphia 5, Pa. 5) National Aluminate Corp., Chicago 38, Ill.
3) Eimer and Amend, New York, N. Y. 6) The Dow Chemical Co., Midland, Mich.

TABLE 3

CHROMATOGRAPHIC ADSORPTION OF AZORUBIN-ALBUMIN MIXTURES ON ANION EXCHANGERS

Adsorbents ground and washed 3 times with water.

Test mixture: 1.0 ml 4.80% Human Albumin in Krebs Buffer (pH 7.8), + 0.25 ml 0.5% Azorubin in 0.6% NaCl (pH 7.8).

The test mixture contains 3.84% albumin, 100 mg % azorubin.

Average values of duplicate or triplicate runs.

Exper. No.	Exchanger No. (See Table 2)	Length of Column in mm	Red Zone in mm from Top	Last 0.25 ml of Filtrate Contained	
				mg % Azorubin	% Albumin*
1	2	34	0-34	9.0	3.63
2	6	30	0-5	0	3.59
3	9	32	0-32**	13.9	3.64
4	13	33	0-6	0	3.54
5	2 coarse	27	0-27	65.4	3.94
6	2 medium	30	0-30	39.3	3.81
7	2 fine	31	0-6	0	4.09

* Total nitrogen x 6.25

** 0-3 mm from top dark red zone

concentration in the mixture should be at least twice as high as the azorubin-binding capacity. For the blank run, 1 ml of serum is mixed with 0.25 ml of 0.6% sodium chloride. The mixtures are poured on the columns immediately after the water (used to wash in the aluminum oxide) ceases to run out of the capillary. The first 1.0 ml of the filtrate is discarded, of the last 0.25 ml (pH 6.1), 2 dilutions (1:20) are made with $\frac{M}{15}$ phosphate buffer, pH 7.7 (0.1 ml of the last part of the filtrate plus 1.9 ml of the phosphate buffer). The optical density is determined at 515 mμ using the Coleman Jr. spectrophotometer and 10 x 75 mm tubes. The 1:20 dilution of the blank is read first against the phosphate buffer in order to have an approximate indication of the serum color which may be considerable in hemolyzed blood samples. It is then used as the blank for the readings of the test solutions. With sera of low ABC (<10 mg %) only one 1:10 dilution is made (0.2 ml plus 1.8 ml phosphate buffer). The azorubin concentrations are read from standard curves prepared as described in Section H. The value obtained, expressed in mg %, is the "ABC observed" of the serum or the

protein solution analyzed. For the characterization of the albumin, this value is divided by the albumin concentration, which is determined electrophoretically. This "specific ABC" value may be expressed in mg per gm albumin, or (after dividing by 502, the molecular weight of azorubin) in 10^{-5} Mol azorubin per gm albumin.

Table 4 shows that the ABC values obtained with the previous and the modified method, using albumin solutions containing various concentrations of azorubin, are essentially the same. The range of error of the method may be seen from Table 5, in which are included all colorimetric values obtained in an experimental series of rats.

TABLE 4

ABC VALUES OF ALBUMIN SOLUTIONS
DETERMINED IN 2 ML AND 1 ML SAMPLES

Albumin Solutions: 4% (approximately) bovine serum albumin (Armour, Fraction V, Lot No. C 1404) in a solution of low molecular inorganic and organic compounds (see reference (1), Table 10).

Azorubin Solutions: In 0.6% NaCl.

2.0 (1.0) ml of the albumin solution was mixed with 0.5 (0.25) ml of an azorubin solution.

Average values of several experiments.

mg % Azorubin in Test Mixture	ABC Observed. Method: 2.5 ml of mixture, 1.0 gm of anionotropic Al_2O_3, Diameter of tubes 8 mm.	ABC Observed. Method: 1.25 ml of mixture, 0.5 gm of anionotropic Al_2O_3, Diameter of tubes 5 mm
100	21.3 mg %	21.5 mg %
80	18.0	16.8
40	17.3	17.5
20	15.8	15.8
10	9.7	9.7
Average value of all Experiments	18.9 mg %	18.9 mg %

TABLE 5

REPRODUCIBILITY OF ABC VALUES

Optical densities of the two 1:20 dilutions of the second filtrates.

Rat sera, pooled.

Mixture of 1.0 ml of serum and 0.25 ml of 0.5% azorubin in 0.6% sodium chloride.

Serum No.	1st Chromato. Run		2nd Chromato. Run		3rd Chromato. Run		Average
	1st Dilution	2nd Dilution	1st Dilution	2nd Dilution	1st Dilution	2nd Dilution	
1	0.169	0.173	0.175	0.171	0.163	0.165	0.169
2*	0.230	0.218	0.222	0.228	-	-	0.225
3	0.248	0.242	0.240	0.232	0.241	0.243	0.241
4*	0.241	0.239	0.245	0.255	-	-	0.245
5*	0.217	0.211	0.211	0.215	-	-	0.214
6*	0.139	0.146	0.137	0.137	-	-	0.140
7	0.212	0.220	0.198	0.215	0.212	0.218	0.215
8	0.269	0.278	0.263	0.268	0.269	0.268	0.269

* Supply limited

E. Fractional Distribution of Azorubin in the Chromatographic Filtrate

In earlier experiments (1) the last part of the chromatographic filtrate was collected for the colorimetric analysis, after a homogenous solution of azorubin was apparent in the capillary. In order to follow quantitatively the distribution of the dye in the filtrate, a series of experiments were done in which the whole filtrate was collected in fractions of 5 drops each. Figure 1 shows the result for two of these runs using a chromatographic tube which delivered 125 drops per 1.25 ml of the azorubin-protein mixture. A comparatively constant value for the optical density was obtained with the 16th tube, i.e., after 0.75 ml had dripped off the column. For the determination of ABC, therefore, it was considered best to discard the first 1.0 ml, and to use the last 0.25 ml of the filtrate for the colorimetric determination.

F. Evidence for a Competition Reaction

The chromatographic procedure for the determination of the ABC value of a protein solution consists of a competitive adsorption of the azo dye by the anionotropic aluminum oxide and the protein. This has been

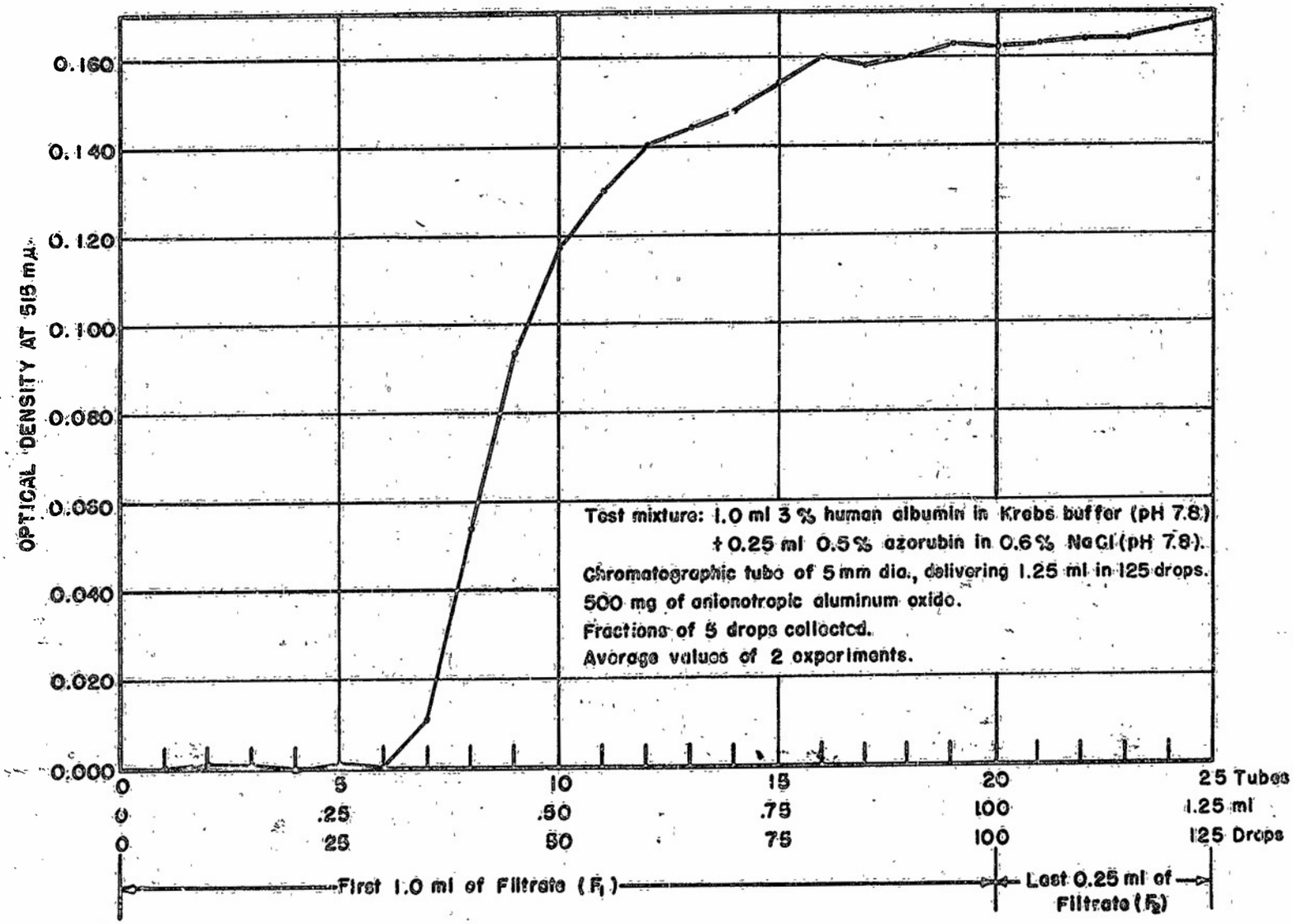


FIG. 1. DISTRIBUTION OF AZORUBIN IN THE CHROMATOGRAPHIC FILTRATE.

demonstrated previously by a comparative estimation of the ABC value of an albumin solution using different amounts of the adsorbent (1). Additional evidence for the competitive reaction has been obtained by directly measuring the specific ABC values of the first (1.0 ml) and second (0.25 ml) part of the chromatographic filtrate. Table 6 demonstrates that the amount of azorubin bound per gram of albumin is markedly lower in the first than in the second part of the chromatographic filtrate. Some azorubin, therefore, is being detached from albumin and adsorbed by the aluminum oxide during the chromatographic process. This affects the results as explained in Section G.

TABLE 6

DETACHMENT OF AZORUBIN FROM ALBUMIN DURING THE CHROMATOGRAPHIC PROCESS

Test Mixture: 1.0 ml albumin in sodium chloride solution + 0.25 ml 0.5% azorubin in 0.6% NaCl.

Original Albumin Solution		0.30% in 0.6% NaCl	0.30% in 0.15 M NaCl	4.19% in 0.15 M NaCl
Test Mixture (1.25 ml)	Albumin concentration	0.24%	0.24%	3.35%
	Azorubin concentration	100 mg %	100 mg %	100 mg %
First Filtrate (1.0 ml)*	Albumin concentration (Kjeldahl)	0.11%	0.09%	2.19%
	Azorubin concentration	0.53 mg %	0.58 mg %	38.2 mg %
	Specific ABC	0.96×10^{-5} Mol	1.28×10^{-5} Mol	3.48×10^{-5} Mol
Second Filtrate (0.25 ml)	Albumin concentration (Kjeldahl)	0.21%	0.19%	3.22%
	Azorubin concentration	2.02 mg %	2.12 mg %	65.0 mg %
	Specific ABC	1.96×10^{-5} Mol	2.22×10^{-5} Mol	4.02×10^{-5} Mol

* The first filtrate includes the water retained in the spaces between the aluminum oxide particles.

G. Influence of Albumin Concentration on the Specific ABC Values. Influence of Non-Binding Protein

It follows from the experiments on the competitive adsorption described in the preceding section that the anionotropic aluminum oxide will detach relatively more azorubin from solutions of low albumin concentration than from solutions of high albumin concentration. The specific ABC value of the second part of the filtrate (0.25 ml), therefore, will be small with low albumin concentrations of the test solutions. In order to learn to what degree this effect influences the ABC, specific ABC values were determined in albumin solutions of different concentrations. Table 7

gives the experimental details and the results. As may be seen from Figure 2, the decrease of the specific ABC values was in the range of the experimental error of 3% for albumin concentrations down to about 2%. At albumin concentrations of 0.3%, as they may occur in severe cases of nephrosis, the specific ABC was found to be about 50% of the values observed for "normal" albumin levels. In cases of extreme hypoproteinemia, therefore, a lowering of the specific ABC values can be considered significant only if the deviation from normal values is greater than the error involved in the method, as indicated in Figure 2. Specific ABC values several hundred per cent lower than normal have been observed in various pathological cases (2).

TABLE 7

INFLUENCE OF ALBUMIN CONCENTRATION ON SPECIFIC ABC VALUES

Solutions of human albumin (recrystallized twice) were prepared in 0.15 M and 0.6 % sodium chloride. 1.0 ml of the albumin solutions was mixed with 0.25 ml of 0.5% azorubin in 0.6% NaCl.

Original Albumin Solution (1.0 ml)		% Albumin in Mixture (1.25 ml)	% Albumin in 2nd Filtrate (0.25 ml)	Decrease in Albumin Concen.	mg % Azorubin in 2nd Filtrate	Specific ABC in 10^{-5} Mol per gm Albumin	% Decrease
Solvent	% Albumin						
0.15 M NaCl	4.19	3.35	3.22	0.13%	65.0	4.02	(0)
"	2.40	1.92	1.80	0.12	35.8	3.96	1.5
"	1.20	0.96	0.87	0.09	15.6	3.57	11.2
"	0.30	0.24	0.19	0.05	11.8	2.31	42.5
0.6% NaCl	3.87	3.10	2.97	0.13	55.5	3.72	(0)
"	1.93	1.55	1.44	0.11	25.6	3.54	4.8
"	0.97	0.78	0.70	0.08	10.1	2.88	22.6
"	0.48	0.38	0.33	0.05	3.9	2.35	36.8
"	0.30	0.24	0.21	0.03	2.0	1.90	48.9
"	0.24	0.19	0.16	0.03	1.1	1.37	63.2

In a mixture of azorubin and serum, the dye is exclusively bound to the albumin component (1, 2). This is in accord with the observations of Klotz, who could not find any binding of certain anions to the serum gamma globulin (4). It was found that the addition of various amounts of a non-binding protein (gamma globulin) to a solution of crystalline human albumin did not alter the specific ABC value of the albumin (see Table 8).

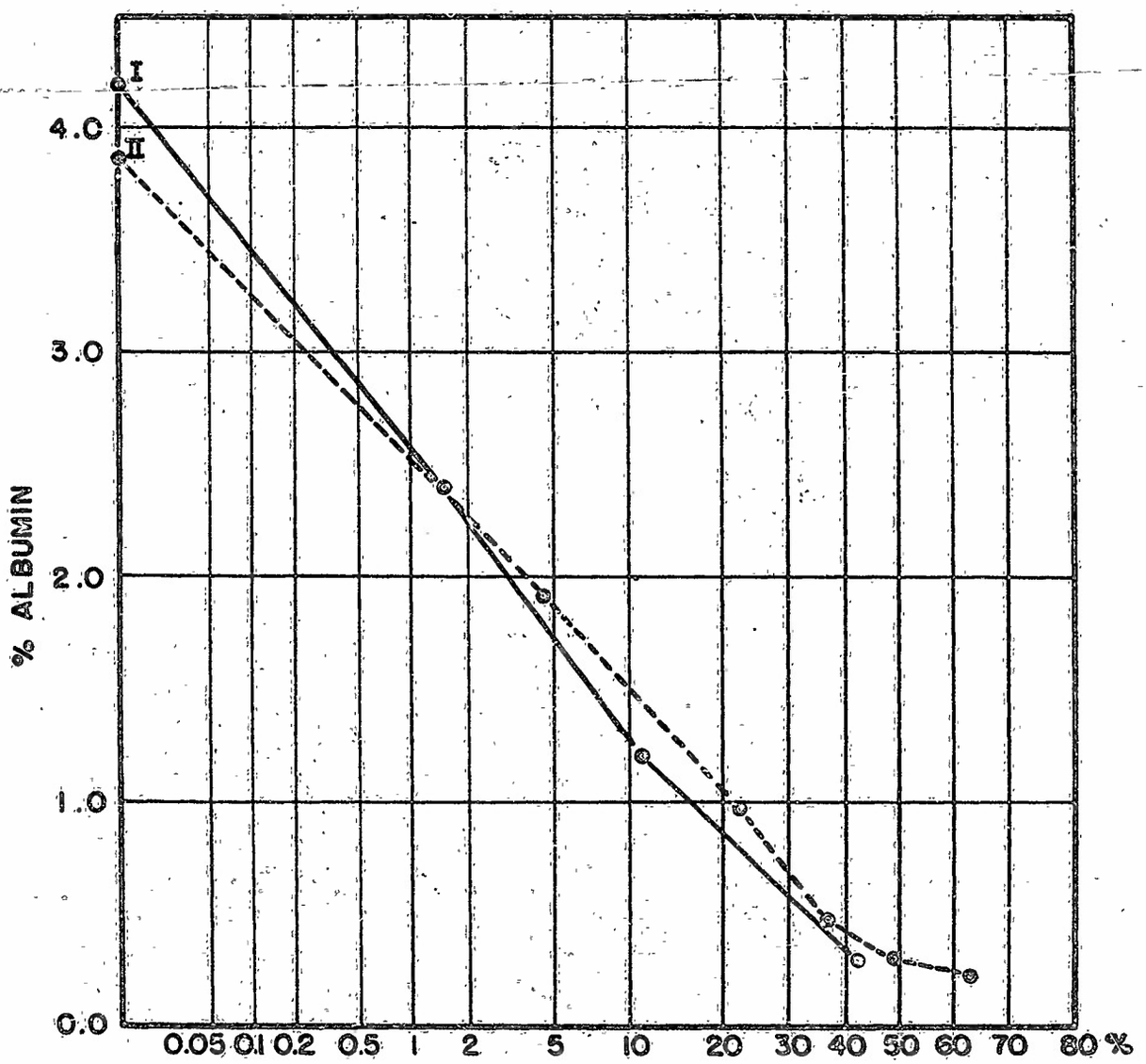


FIG. 2. % DECREASE OF SPECIFIC ABC WITH DECREASING ALBUMIN CONCENTRATIONS

I Crystalline Human Albumin in 0.15 M Sodium Chloride.
II Crystalline Human Albumin in 0.6 % Sodium Chloride.

TABLE 8

INFLUENCE OF GAMMA GLOBULIN ON THE SPECIFIC ABC OF ALBUMIN			
Protein Preparations: Human Albumin, Recrystallized Twice, Human Gamma Globulin			
1 ml of Protein Solution		0.25 ml of Azorubin Solution in 0.6% NaCl Containing	Specific ABC in 10^{-5} Mol Azo/gm Albumin
Containing	In		
1.20% Albumin	0.15 M NaCl	500 mg % Azorubin	3.57
1.20% Albumin & 4.66% gamma globulin	"	"	3.58
2.06% Albumin	Krebs Buffer pH 7.8	500 mg % Azorubin	2.55
2.06% Albumin & 1.61% gamma globulin	"	"	2.50

H. Calibration Curves for Azorubin Bound to Albumin of Different Species

The optical properties of acidic and basic dyes may be altered by combining them with proteins. The studies of Klotz (4) have shown that the extinction coefficient of several anionic azo dyes is decreased by the addition of pure bovine serum albumin. For the estimation of the azorubin concentration in the second chromatographic filtrate (see above), it is therefore necessary to prepare calibration curves using mixtures of the azorubin with the same proteins for which the ABC values are to be determined. Concentrations of these proteins and azorubin, as well as other conditions, must be approximately the same as in the actual test procedure. The solutions of the albumin preparations and the sera were diluted 4:5 by adding $\frac{1}{4}$ volume of the azorubin solutions. These mixtures were diluted 1:10 or 1:20 respectively with $\frac{M}{15}$ phosphate buffer at pH 7.7, and their extinction measured as described in Section D. The calibration curves are given in Figure 3 and 4. As is to be expected, an individual calibration curve must be used for sera or albumin preparations of any species.

I. ABC Value of Bovine Serum

Bovine serum was analyzed for total protein, non-protein nitrogen, and albumin by the Kjeldahl procedure as modified by Van Slyke and associates (5). For separating the globulins from the albumin fraction, the 26.8% Na_2SO_4 precipitation procedure (6, 7) was used. Since azorubin has been found to be bound exclusively to albumin as characterized by electrophoresis (1), the determination of albumin in the bovine serum was done by electrophoretic analysis. The serum was dialyzed for 3 days against Michaelis buffer, pH 8.0,

FIGURE 3

Calibration Curves for Azorubin in Solutions of Human and Bovine Serum Albumin

Protein Solutions used for the Calibration Curves:

I. 4.19% Human Serum Albumin (recrystallized twice) in 0.15 M sodium chloride.

II & III. 4.0% Bovine Serum Albumin (Armour, Fraction V, Lot #C 1404), in a solution of inorganic and organic low molecular compounds (see reference (1), Table 10).

For the azorubin solutions, a preparation refined as described in Section A was dissolved in 0.6% sodium chloride.

The albumin-azorubin mixtures were diluted 1:10 (II) or 1:20 (I, III) with $\frac{M}{15}$ phosphate buffer pH 7.7. The colorimetric measurements were made at 515 mμ using the Coleman Jr. spectrophotometer and 10 by 75 mm tubes. Colorimeter blanks were prepared using all components except azorubin.

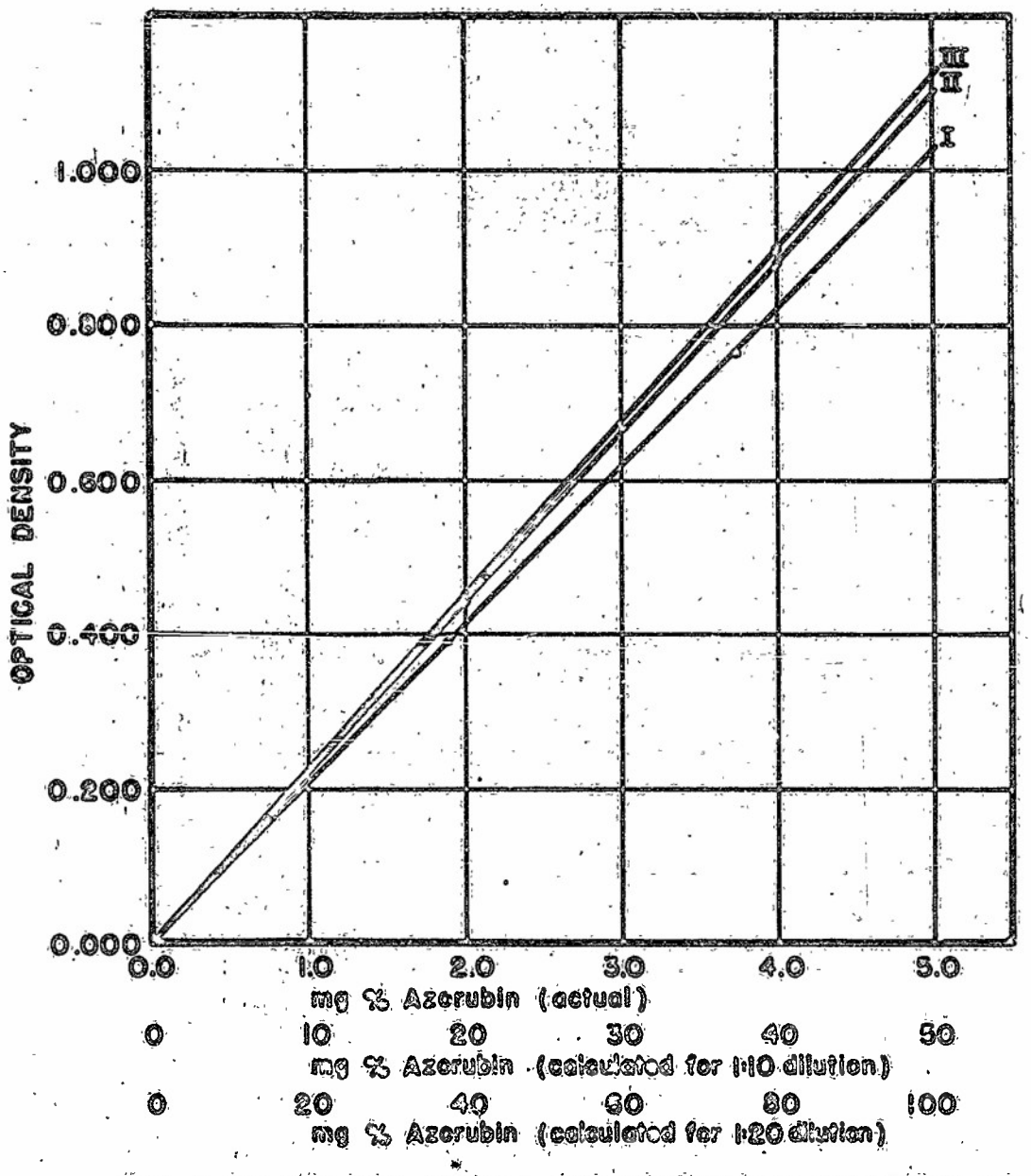


FIG. 3 CALIBRATION CURVES FOR AZORUBIN IN SOLUTIONS OF HUMAN AND BOVINE SERUM ALBUMIN.

FIGURE 4

Calibration Curves for Azorubin in Sera of Ox, Rat, and Rabbit

Procedure as described in Figure 3.

Sera used for calibration curves:

I & II. Bovine serum obtained from the slaughter house, Louisville.

III. Pooled serum of 3 normal rats, average weight 400 gm. Average of 2 determinations.

IV. Sera of 4 normal rats, average weight 399 gm. Average of 9 determinations.

V. Sera of 4 normal rabbits, average weight 2450 gm. Average of 8 determinations.

The albumin-azorubin mixtures were diluted 1:10 (I, III) or 1:20 (II, IV, V).

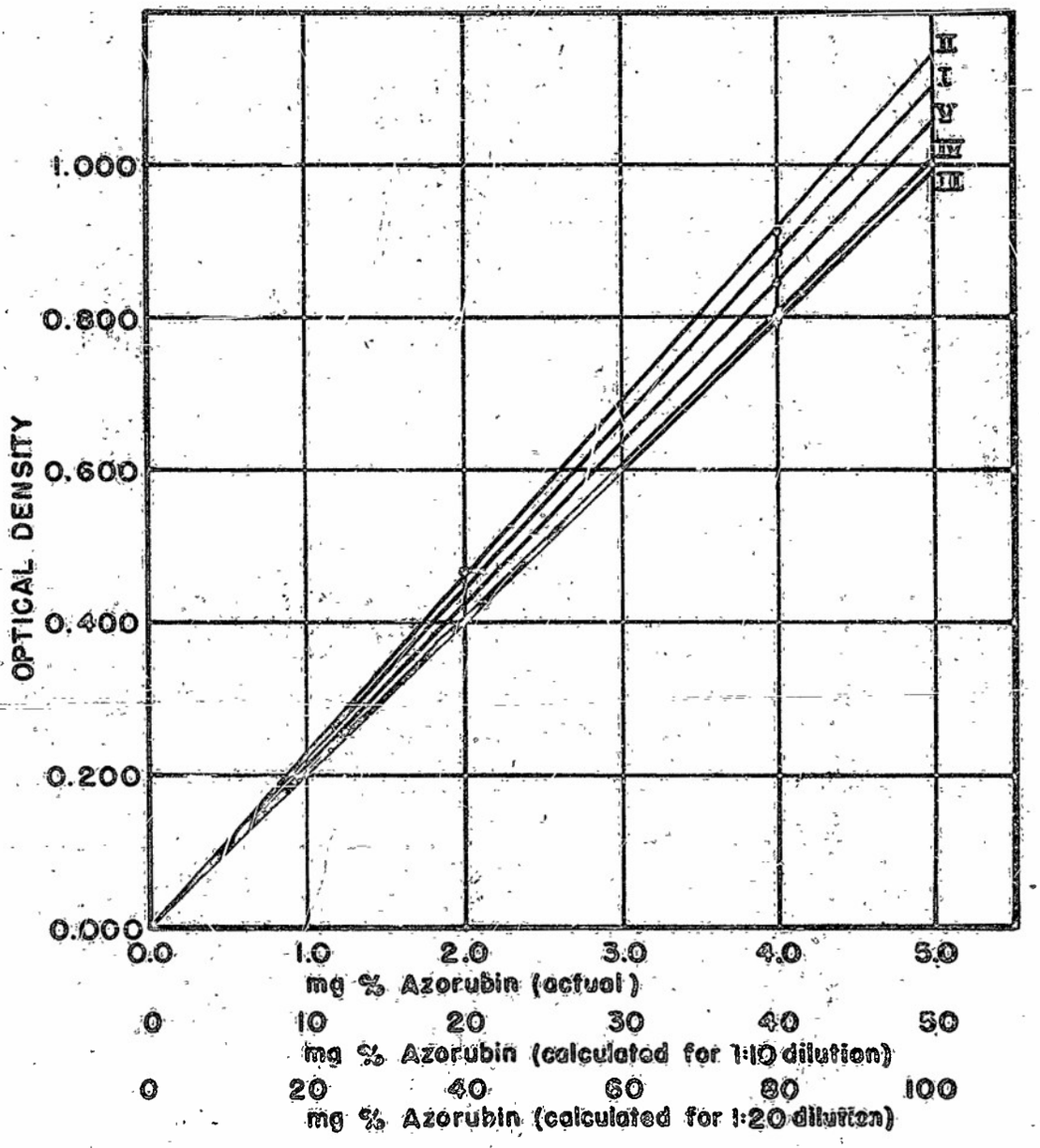


FIG. 4 CALIBRATION CURVES FOR AZORUBIN IN SERA OF OX, RAT, AND RABBIT.

ionic strength 0.1, at 2-3°C. The electrophoretic analysis was made using the "compact" apparatus of the Perkin-Elmer Corporation (8).* The albumin content was calculated from the average planimetric values of the ascending and descending boundaries. Electrophoretic and chemical albumin values were found to be in good agreement; the specific ABC was calculated using both values (Table 9).

TABLE 9

ANALYSIS OF BOVINE SERUM			
Analysis of	Average Value	No. of Experiments	Method
Total Protein	7.20%	8	Kjeldahl (5)
Non-Protein Nitrogen	22.8 mg %	6	Uranium acetate; Kjeldahl (5)
Albumin	3.56% (49.4%)	6	Majoor (6) Kjeldahl (5)
Albumin	(3.58%) 49.7%	1	Electrophoresis (8)
ABC observed	20.0 mg %	5	Chromatographic Method
Specific ABC	1.40×10^{-5} Mol	-	Calculated from chemical albumin
Specific ABC	1.39×10^{-5} Mol	-	Calculated from electrophoretic albumin

III. SUMMARY

1. A modified method has been described for the determination of the azorubin-binding capacity (ABC), using only 1 ml of serum or protein solution. The conditions for this procedure have been investigated. The new method gives the same results as the previous procedure (1), but has the advantage of using smaller amounts of serum.

2. A process for a graded deactivation of anionotropic aluminum oxide has been developed.

* The electrophoretic analysis was done by Mr. R. DeArmond of this laboratory.

3. Attempts to replace the anionotropic aluminum oxide by synthetic anion exchangers were unsuccessful. This is believed to be due to the slow diffusion of large anions such as azorubin into the solid resins. This process influences the degree of retention. Employing anionotropic aluminum oxide, the anions are mainly adsorbed at the active centers of the surface.

4. The competitive reaction between anionotropic aluminum oxide and albumin for azorubin has been further investigated. A quantitative relationship has been established between the albumin concentration and the specific ABC values.

5. The ABC of a serum albumin solution is not influenced by the addition of a non-binding protein (gamma globulin).

6. Calibration curves are given for the determination of azorubin bound to serum albumin of different species.

7. ABC values of bovine serum are presented.

IV. CONCLUSIONS

The present chromatographic method for a comparative determination of the ABC of serum albumin can be applied to studies on small laboratory animals. In the interpretation of the results, the influence of low albumin concentrations on the specific ABC must be considered.

V. RECOMMENDATIONS

The modified method can be used in determining the specific ABC of the serum of animals subjected to various forms of stress as well as experimental liver damage.

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